

# IOWA STATE UNIVERSITY

## Digital Repository

---

Entomology Publications

Entomology

---

2016

## A Review of Cry Protein Detection with Enzyme-Linked Immunosorbent Assays

Vurtice C. Albright III

*Iowa State University*

Richard L. Hellmich

*U.S. Department of Agriculture, rlhellmi@iastate.edu*

Joel R. Coats

*Iowa State University, jcoats@iastate.edu*

Follow this and additional works at: [https://lib.dr.iastate.edu/ent\\_pubs](https://lib.dr.iastate.edu/ent_pubs)



Part of the [Agriculture Commons](#), [Cell and Developmental Biology Commons](#), [Entomology Commons](#), [Environmental Sciences Commons](#), and the [Genetics Commons](#)

The complete bibliographic information for this item can be found at [https://lib.dr.iastate.edu/ent\\_pubs/492](https://lib.dr.iastate.edu/ent_pubs/492). For information on how to cite this item, please visit <http://lib.dr.iastate.edu/howtocite.html>.

---

This Article is brought to you for free and open access by the Entomology at Iowa State University Digital Repository. It has been accepted for inclusion in Entomology Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact [digirep@iastate.edu](mailto:digirep@iastate.edu).

---

# A Review of Cry Protein Detection with Enzyme-Linked Immunosorbent Assays

## Abstract

The widespread use of Cry proteins in insecticide formulations and transgenic crops for insect control has led to an increased interest in the environmental fate of these proteins. Although several detection methods are available to monitor the fate of Cry proteins in the environment, enzyme-linked immunosorbent assays (ELISAs) have emerged as the preferred detection method, due to their cost-effectiveness, ease of use, and rapid results. Validation of ELISAs is necessary to ensure accurate measurements of Cry protein concentrations in the environment. Validation methodology has been extensively researched and published for the areas of sensitivity, specificity, accuracy, and precision; however, cross validation of ELISA results has been studied to a lesser extent. This review discusses the use of ELISAs for detection of Cry proteins in environmental samples and validation of ELISAs and introduces cross validation. The state of Cry protein environmental fate research is considered through a critical review of published literature to identify areas where the use of validation protocols can be improved.

## Keywords

bioassays, cross validation, Bt proteins, Cry protein, ELISA, environmental fate, transgenic crops

## Disciplines

Agriculture | Cell and Developmental Biology | Entomology | Environmental Sciences | Genetics

## Comments

This article is published as Albright III, Vurtice C., Richard L. Hellmich, and Joel R. Coats. "A review of Cry protein detection with enzyme-linked immunosorbent assays." *Journal of agricultural and food chemistry* 64, no. 11 (2016): 2175-2189. doi: [10.1021/acs.jafc.5b03766](https://doi.org/10.1021/acs.jafc.5b03766).

## Rights

Works produced by employees of the U.S. Government as part of their official duties are not copyrighted within the U.S. The content of this document is not copyrighted.

# A Review of Cry Protein Detection with Enzyme-Linked Immunosorbent Assays

Vurtice C. Albright, III,<sup>†</sup> Richard L. Hellmich,<sup>‡</sup> and Joel R. Coats<sup>\*,†</sup>

<sup>†</sup>Pesticide Toxicology Laboratory, Department of Entomology, Iowa State University, 110 Insectary, Ames, Iowa 50011, United States

<sup>‡</sup>Corn Insects and Crop Genetics Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 110 Genetics Laboratory, Ames, Iowa 50011, United States

## S Supporting Information

**ABSTRACT:** The widespread use of Cry proteins in insecticide formulations and transgenic crops for insect control has led to an increased interest in the environmental fate of these proteins. Although several detection methods are available to monitor the fate of Cry proteins in the environment, enzyme-linked immunosorbent assays (ELISAs) have emerged as the preferred detection method, due to their cost-effectiveness, ease of use, and rapid results. Validation of ELISAs is necessary to ensure accurate measurements of Cry protein concentrations in the environment. Validation methodology has been extensively researched and published for the areas of sensitivity, specificity, accuracy, and precision; however, cross validation of ELISA results has been studied to a lesser extent. This review discusses the use of ELISAs for detection of Cry proteins in environmental samples and validation of ELISAs and introduces cross validation. The state of Cry protein environmental fate research is considered through a critical review of published literature to identify areas where the use of validation protocols can be improved.

**KEYWORDS:** bioassays, cross validation, Bt proteins, Cry protein, ELISA, environmental fate, transgenic crops

## ■ INTRODUCTION TO Cry PROTEINS

**Origin.** Insecticidal crystalline proteins were first discovered, unknowingly, in 1901 when S. Ishiwata isolated a micro-organism from a diseased silkworm larva (*Bombxy mori*), which he named *Bacillus sotto*.<sup>1</sup> [Although initially discovered and named by Ishiwata, because he did not formally describe it, Ernst Berliner received credit for naming it *Bacillus thuringiensis* when he discovered a similar microorganism in diseased Mediterranean flour moth larvae (*Anagasta kuehniella*) living in stored grain in the state of Thuringia, Germany in 1911.]<sup>2</sup> In a follow-up report in 1905 Ishiwata noted "death occurs before the multiplication of the bacillus..." where the first indication that a toxin is at least partially responsible for the pathogenicity of *B. thuringiensis* arises.<sup>2,3</sup> However, identification of the toxic agent had to wait until 1954 when T. A. Angus showed that bipyramidal crystals present in sporulating *B. thuringiensis* cells were actually responsible for toxicity to *Bombxy mori* larvae, while spores alone had no effect.<sup>2,4</sup> From the 1960s to the 1980s, new *B. thuringiensis* subspecies such as *kurstaki*, *kurstaki* HD-1, and *tenebrionis* were identified, bringing with them new crystalline proteins or Cry protein.<sup>2</sup> Currently, there are more than 70 primary classes of Cry proteins (complete list available at [www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/intro.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html)).

**Mechanism of Action.** The exact mechanism of action of Cry protein toxins is not currently known, but it is generally recognized as a multistep process. Three different proposed models of mechanism of action are described in detail elsewhere.<sup>5–7</sup> For the purposes of this review, only a summary of the basic mechanism of action steps is provided. First, the Cry proteins must be ingested by a susceptible species, as they have no contact toxicity.<sup>8</sup> Once inside the insect midgut, the Cry proteins are solubilized in an alkaline (for lepidopteran and dipteran insects) or a neutral or acidic (for coleopteran insects)

environment.<sup>8</sup> Proteases from the host insect, such as trypsin and chymotrypsin, then process the Cry proteins, cleaving off portions of the N- and C-terminals, leaving only the activated toxin.<sup>9</sup> The activated toxin binds to specific receptors on the midgut epithelium and destabilizes the epithelium cells, causing them to swell and resulting in cell lysis.<sup>5–8</sup> The loss of several epithelium cells compromises the insects midgut and results in the death of the insect.<sup>5–7</sup>

**Usage as Insecticides and Development of Transgenic Traits.** Insecticidal formulations containing a mixture of *Bacillus thuringiensis* spores and Cry proteins have been used to control pest insects for decades. Although the first commercial insecticide product was introduced in France in 1938 to target lepidopteran pests, it was not until the introduction of Thuricide in the late 1950s that these formulations were used on a wide scale.<sup>10,11</sup> Sprays, powders, and other formulations of Cry proteins or the bacterium *B. thuringiensis* continue to be used in agricultural production on field crops such as cotton, potatoes, and maize, and on nonfield crops such as fruit trees, avocados, and strawberries.<sup>12–14</sup> Cry protein-based insecticides also have found uses outside of agriculture including controlling for forest pests, nematodes, and termites.<sup>15–17</sup> In addition to plant protection, Cry protein-based insecticides also can be used in protection of humans. Formulations containing Cry proteins also are used to control black flies in West Africa (prevention of spreading of disease-causing parasitic worms) and Argentina and Brazil (as nuisance control).<sup>18–20</sup> Cry protein-based insecticide formulations have been developed to control

**Received:** July 31, 2015

**Revised:** February 26, 2016

**Accepted:** March 7, 2016

**Published:** March 7, 2016

mosquitoes, as they are vectors for some of the most significant human diseases, such as malaria, dengue, and lymphatic filariasis.<sup>18</sup> These formulations are used throughout Asia, Europe, and North and South America.

Currently, Bt formulations are primarily used by gardeners, organic farmers, and in forestry.<sup>11</sup> Their widespread use in agriculture has been hindered by the narrow specificity of the formulations, a lack of systemic activity, and a lack of stability in the environment, resulting in low residual activity.<sup>11</sup> These factors can trigger the need for multiple applications to ensure protection is achieved, and such near-constant spraying is not economically viable.<sup>21</sup> Another limitation in the use of Cry protein formulations in agriculture has been the development and rapid increase in the use of transgenic plants producing Cry proteins for protection against pest insects. Development of transgenic plants expressing genes for the production of Cry proteins occurred near simultaneously in multiple laboratories. Fischhoff et al.<sup>22</sup> developed transgenic tomato plants for the control of several lepidopteran pests. These plants achieved 50–100% mortality of tobacco hornworm larvae (*Manduca sexta*).<sup>22</sup> Meanwhile, other laboratories were developing transgenic tobacco plants also to control for *M. sexta*. Vaeck et al. developed transgenic tobacco plants that cause 100% mortality of *M. sexta* larvae after 6 days.<sup>23</sup> Barton et al.<sup>24</sup> also developed transgenic tobacco plants for protection against *M. sexta*, using a different trait, that were capable of achieving mortality at levels similar to those observed in the previous two studies.<sup>24</sup> Following up on these successes, other transgenic crops were quickly developed, including cotton, rice, potato, and maize.<sup>25–28</sup>

All of this research led to the approval in 1995 of the first transgenic crops conferring insect resistance for commercial use.<sup>29</sup> Included in the first round of approvals was Maximizer maize (Ciba-Geigy), Bollgard cotton and New Leaf potatoes (both Monsanto products), all of which were first planted commercially in 1996.<sup>29,30</sup> Initially, only traits for cotton, maize, and potato were available. Since these initial introductions, transgenic cotton and maize expressing Cry proteins for insect resistance have been widely adopted, while transgenic potatoes have not. As of 2015, 81% of maize and 84% of cotton planted in the United States expressed one or more insecticidal traits for protection against a variety of insect pests.<sup>31</sup> Sales of New Leaf potato seeds never rose above 5% of the total amount of potato seed sold, and it was eventually pulled from the market after six years.<sup>32</sup>

Although maize and cotton are the only transgenic crops expressing insecticidal traits currently available in the U.S., other crops are in various stages of development. Development of transgenic tomato, tobacco, and rice plants expressing genes for production of insecticidal Cry proteins have already been mentioned in this review. However, these research efforts have yet to produce any commercially available products.<sup>30,33</sup> Other transgenic crops with Cry proteins for insect protection have been developed and have even been approved for commercial usage in other countries. Transgenic soybean plants have been engineered to produce Cry1Ac for the control of several insect pests.<sup>34,35</sup> Transgenic soybean producing Cry1Ab protein was registered for commercial use in Brazil in 2011.<sup>36</sup>

Several other vegetable species also have been genetically modified to produce Cry proteins for insect protection. The most successful, and only one currently in commercial usage, is sweet corn engineered to produce Cry1Ab. It was first introduced in 1998 by Novartis Seeds and initially struggled to gain a large market share due to a lack of public acceptance;

however, it is currently estimated that Bt sweet corn accounts for 18–25% of the total fresh sweet corn market.<sup>37,38</sup> A transgenic brinjal (eggplant) was approved for commercialization in India in 2009, but it never reached market due to significant political pressure.<sup>39</sup> However, it was released to a limited number of farmers in Bangladesh in early 2014.<sup>40</sup> Numerous *Brassica* species have been developed including cabbage, broccoli, cauliflower, canola, and rutabaga.<sup>41–45</sup> Despite the extensive research into these transgenic *Brassica* vegetables, viable commercial products have not yet been developed.

Two nontraditional crop plants under development that produce insecticidal Cry proteins are poplar and eucalyptus trees. Poplar trees are important to the paper and timber industries due to their rapid growth rate, but are susceptible to damage from a variety of coleopteran and lepidopteran pests.<sup>46,47</sup> Transgenic poplar trees have been developed to provide protection against either coleopteran or lepidopteran insect pests, resulting in significant protection.<sup>46,47</sup> Like poplar trees, eucalyptus trees are important sources for timber and pulp for paper, primarily in Australia, and are susceptible to defoliation; transgenic eucalyptus trees have been shown to have excellent protection against insects.<sup>48</sup>

**Nontarget Toxicity Concerns.** Although individual Cry proteins have a narrow spectrum of activity against specific insect orders, some nontarget effects have been reported. Much work has focused on monarch butterflies (*Danaus plexippus*) after a paper by Losey et al.<sup>49</sup> reported decreased growth rates and survival of monarchs fed with pollen from transgenic Bt corn in a laboratory study and Jesse and Obrycki<sup>50</sup> reported lethal effects from a field study. Follow-up studies determined only one type of Bt maize (Event 176), which is no longer commercially available, was lethal to monarch larvae.<sup>51–53</sup> Effects from other types of commercially available maize were expected to be minimal due to low toxicity and low exposure.<sup>54</sup> Continuous exposure of larvae to pollen from Cry1Ab Bt maize throughout development, a worse-case exposure scenario, found evidence of reduced feeding, decreased weight gain, longer development time, and increased larval mortality.<sup>53</sup> The estimated additional mortality to monarch population due to worse-case exposure was only 0.6%.<sup>53</sup>

Other nontarget insects in the order Lepidoptera also have been reported to be susceptible to Cry1Ab. The lycaenid butterfly *Pseudozizeeria maha* fed on Cry1Ab-containing pollen in laboratory studies exhibited decreased larval survival.<sup>55</sup> Higher mortalities and decreased weight gain also have been reported for *Pieris brassicae*, *Pieris rapae*, and *Plutella xylostella* in laboratory studies.<sup>56</sup> It should be noted, however, that both studies utilized pollen from maize event 176, which is no longer commercially available.

Some closely related Trichopteran species have been reported to be susceptible to lepidopteran-active Cry proteins. Decreased growth rates have been reported for the caddis fly *Lepidostoma liba* fed transgenic maize detritus; the detritus was not analyzed, so the quantity and quality of the Cry proteins to which the larvae were exposed were unknown<sup>57,58</sup> (although subsequent studies refute this claim<sup>59</sup>). Increased mortality and decreased abundance in fields where Cry1Ab was present have been reported for *Helicopsyche borealis* and *Pycnopsyche* sp. respectively.<sup>57,60</sup> Effects on nontarget Dipterans have been reported with the aquatic midge *Chironmus dilutus* showing decreased survival on Cry3Bb1 and the crane fly *Tipula abdominalis* exhibiting decreased growth rates on transgenic Cry1Ab maize (though the latter may be due to tissue differences



between transgenic maize and near-isoline nontransgenic maize).<sup>59,61</sup> Cry1Ac has been reported to be more toxic to *C. dilutus* than to *Hyalella azteca*, but this toxicity is limited at environmentally relevant concentrations.<sup>62</sup> [Further reading on the effects of Cry proteins to nontarget organisms has been reviewed elsewhere.<sup>63–65</sup>]

**Environmental Chemistry.** As a result of these potential nontarget toxicity issues, it is important to address questions surrounding the environmental fate of Cry proteins. Environmental fate questions that must be addressed include, but are not limited to degradation, persistence, mobility, and bioavailability of the Cry proteins. However, before any of these questions can be answered, researchers must have adequate tools for detecting and quantifying the amount of Cry proteins in the environment. There are currently a wide variety of detection methods available for Cry proteins, including high-performance liquid chromatography/mass spectrometry, biological assays (bioassays), and Western blotting. A drawback of these methods is that they are time- and labor-intensive and as a result, are generally cost-prohibitive for most researchers. Thus, most researchers elect to use enzyme-linked immunosorbent assays (ELISAs) to detect Cry proteins in environmental matrices.

## ■ ELISA USAGE FOR CRY PROTEIN DETECTION

**Uses in Agriculture.** One of the first studies to investigate the use of enzyme-linked immunosorbent assays (ELISAs) for detecting Cry proteins was by Wie et al.<sup>66</sup> The authors investigated the prospect of using ELISAs to detect and quantify the amount of crystal toxins from *B. thuringiensis* subspecies. They determined that the ELISA method was highly accurate and extremely sensitive. While specificity between dipteran-active and lepidopteran-active toxins could be achieved, there was significant cross-reactivity between lepidopteran subspecies of *B. thuringiensis*.<sup>66</sup> Since that initial study, however, significant amounts of research have been devoted to improving the antibodies used and the ELISA procedure as a whole.<sup>67–69</sup> Early use of ELISAs for detection of Cry proteins required developing antibodies in the laboratory for the specific protein of interest.<sup>70</sup> More recently, commercial manufacturers have been developing ready-made kits with antibodies specific to 1–2 Cry proteins. Supplemental Table 1 contains a list of ready-made ELISA kits available from several manufacturers. [Discussion of ELISA types, antibody types, and ELISA procedures has been covered extensively elsewhere.<sup>71–74</sup>]

There are a wide range of uses for ELISAs currently in agriculture. ELISAs can be used to screen cell cultures or plants for the presence of a novel protein of interest to determine which cell culture or plant is expressing the protein after genetic transformation in a laboratory. These cell cultures or plants can then be further screened to remove cultures or plants that are expressing the protein at insufficient levels. As trials expand into greenhouses or field settings, ELISAs can be utilized in event selection to identify low trait expressing plants, allowing them to be culled from the gene pool.<sup>75</sup> Once a product is ready for commercial usage, ELISAs can be used to determine protein expression levels in roots, leaves, pollen, and other various plant tissues. Such information is typically required by regulatory agencies as part of the extensive registration packages. ELISAs also may be employed as quality assurance/quality control tools during seed production to ensure the products (i.e., seeds) being delivered to the customers will perform as promised.<sup>76</sup> Following harvest, ELISAs can be used to identify transgenic crops from nontransgenic crops and ensure that transgenic

crops do not become mixed with nontransgenic crops throughout the supply and processing chain, where required by governmental regulations.<sup>77</sup>

A major use of ELISAs in agriculture research, and the focus of the remainder of this paper, is for monitoring of Cry proteins in environmental matrices pre- and postharvest. ELISAs are the most popular method currently used to detect Cry proteins in environmental samples. As with any pesticide, insecticides containing Cry proteins may enter soil and water matrices through direct application, such as foliar sprays and soil drenches, or indirect application, such as spray drift and spills. Cry proteins also may move into water via surface water runoff and soil erosion. Another pathway that Cry proteins in transgenic crops can enter the ecosystem is through the transportation of crop material. This typically occurs postharvest as crop residues are incorporated into the soil or transported to water bodies (via wind or surface water runoff) and begin to degrade, releasing Cry proteins.<sup>57,78,79</sup> Cry proteins also may enter soil and water bodies through exudation from plant roots or via pollen deposition.<sup>49,80</sup> Understanding the movement and fate of Cry proteins in the environment is crucial in determining the risk of Cry proteins to nontarget organisms.

Presence/absence, dissipation, persistence, and partitioning of Cry proteins in soil and water matrices can all be addressed by ELISAs for both insecticide formulations containing Cry proteins and transgenic crops producing Cry proteins. In lab studies, Douville et al.<sup>81</sup> spiked soil and water samples with pure Cry1Ab protein to determine persistence in the environment. Aquatic environments degraded the protein more rapidly than soils, with half-lives of 4 and 9 days, respectively.<sup>81</sup> This result was corroborated by other research groups. In a study on the aquatic fate of Cry3Bb1, half-lives of Cry3Bb1 protein in various maize tissues in aquatic microcosms were less than 3 days, and no Cry3Bb1 was detected in the water or sediment.<sup>61</sup> A related study looked at dissipation of Cry3Bb1 in maize tissues in soils and determined there was a slightly longer half-life for the various maize tissues in soil.<sup>82</sup> Another research group showed that two different formulations containing Cry4, a mosquitocidal protein, were below the limit of detection (LOD) of the ELISA after 7 days (LOD = 2 ppb) in aquatic microcosm studies.<sup>69</sup>

In field studies, the information on persistence and dissipation is mixed. Sundaram et al.<sup>83</sup> investigated persistence of a commercial *Bt kurstaki* formulation Foray 48B, on oak leaves following foliar application to protect against the gypsy moth *Lymantria dispar* L. Their results indicated that the amount of toxin present was below the limit of quantification of 8 ppb after only 2 days.<sup>83</sup> Gruber et al.<sup>84</sup> studied the presence of Cry1Ab in soil in fields following cultivation of transgenic maize in the same four fields for nine consecutive years. The authors found no evidence for accumulation or persistence of Cry1Ab during long-term cultivation as only one site had protein levels above the limit of detection 6 weeks after harvest, and no protein was detected at any of the sites the following spring.<sup>84</sup> Daudu et al.<sup>85</sup> tracked the degradation of Cry1Ab in leaf and stem residues in litter bags buried in soils and found that, after 14 days, less than 0.02% of the Cry1Ab remained in the leaf and stem residues. In addition, no Cry1Ab protein was detected in the soil around the litter bags, indicating that the Cry protein was rapidly degraded.<sup>85</sup>

In contrast, Baumgarte and Tebbe<sup>86</sup> studied the amount of Cry1Ab protein in soil and plant tissue residues in two agricultural fields where transgenic maize was grown. Cry1Ab was detected in soils and plant tissues at both field sites during

Table 1. Summary of the ELISA Validation Steps Performed in Studies on the Environmental Fate of Cry Proteins

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Cross Validation
Head et al. <sup>109</sup>	Cry1Ac	Soil	LOD = 3.68 ng/g; LLOQ not reported	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 24–39% depending on soil type; %CV = 21.2% <sup>a</sup>	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Performed bioassays with a susceptible species; bioassays results indicated no protein was present in all samples, which confirmed the ELISA results
Hopkins and Gregorich <sup>129</sup>	Cry1Ab	Soil	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	No recovery methods or data reported; efficiency of extraction procedure unknown	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Zwahlen et al. <sup>108</sup>	Cry1Ab	Maize detritus	LOD, LLOQ not reported	Analyzed maize detritus from non-Bt plants in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Extraction efficiency for maize detritus not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Performed bioassays with a susceptible species; bioassay results indicated decreasing mortality and sublethal effects over time, confirms ELISA results
Ahmad et al. <sup>118</sup>	Cry3Bb1	Soil	Kit LOD = 1 ng/mL; LOD, LLOQ for soil recovery method not reported	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 17–66% depending on spiking solution and soil type; standard error reported, but insufficient information to calculate %CV	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Baumgarte and Tebbe <sup>87</sup>	Cry1Ab	Soil, maize tissue	LOD = 0.19 ng/g soil after adjusting for recovery percentage; LLOQ not reported	Analyzed soils and maize tissue from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Recovery values ranged 34–40% in soil; standard error reported, but insufficient information to calculate %CV; extraction efficiency for maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active; authors recognized this data gap
Wang et al. <sup>126</sup>	Cry1Ab	Rice tissue, soil	LOD = 0.5 ng/g soil, not reported for rice tissue; LLOQ not reported	Analyzed non-Bt rice tissue in parallel, no false positives, indicates cross-reactivity not significant; cross-reactivity and interference in soil not reported	Mean recoveries ranged 46–82% depending on soil type; %CV ranged from 6.1% to 35.1% <sup>b</sup> depending on soil type; extraction efficiency for rice tissue not reported	Used commercial ELISA kit; additional precision data not reported	Performed with susceptible species for rhizosphere soils, no significant effects observed, confirms ELISA results; not performed for soil amended with rice tissue, unknown if protein detected was fully intact and/or biologically active
Marchetti et al. <sup>128</sup>	Cry1Ab, Cry1Ac	Soil	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	Mean recoveries of 67% in clay loam soil, 82% in sandy soil; %CV not reported	Used commercial ELISA kit; additional precision data not reported	Performed with a susceptible species; bioassay results indicated that the decrease in toxicity occurred more rapidly than shown by ELISA results
Nguyen and Jehle <sup>127</sup>	Cry1Ab	Maize tissue, detritus	LOD = 0.14 ng; LLOQ not reported	Analyzed nontransgenic, isoline maize in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active, authors recognized this data gap
Gruber et al. <sup>117</sup>	Cry1Ab	Soil	LOD = 0.4 ng/g soil; Decision Limit (2 ng/g soil) and Detection Capacity (3.1 ng/g soil) determined according to European Commission Decision 2002/657/EC <sup>34</sup>	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 45–91% depending on soil type %CV not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Not performed, but no protein detected in any soil samples potentially renders bioassays unnecessary

Table 1. continued

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Cross Validation
Icoz and Storzky <sup>119</sup>	Cry3Bb1	Maize tissue, root extracts, soil	Kit LOD = 1 ng/mL; LOD, LLOQ for soil recovery method not reported	Cross-reactivity and interference due to matrix components not reported	Recovery from soil and root extracts and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Prihoda and Coats <sup>62</sup>	Cry3Bb1	Soil, maize tissue	LOD = 0.7 ng/mL; LLOQ = 9 ng/g soil	Analyzed nontransgenic, isoline maize in parallel, no false positives; indicates cross-reactivity not significant; interference not reported	Mean recovery decreased from 41% (0 h) to 15% (96 h); %CV ranged from 6.1% to 35.1%; <sup>b</sup> extraction efficiency from maize tissue not reported	Used commercial ELISA kit; reanalyzed plates with %CV > 10%	Not performed; unknown if protein detected was fully intact and/or biologically active
Prihoda and Coats <sup>61</sup>	Cry3Bb1	Water, sediment, maize tissue	LOD, LLOQ not reported	Analyzed nontransgenic, isoline maize in parallel, no false positives; indicates cross-reactivity not significant; interference not reported	Water analyzed directly; recovery from sediment and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; reanalyzed plates with %CV > 10%	Not performed; unknown if protein detected was fully intact and/or biologically active
Schrader et al. <sup>130</sup>	Cry1Ab	Soil, maize detritus, earthworm tissue	LOD = 0.017 ng/g soil, 1 ng/g maize detritus, 0.58 ng/g earthworm tissue; LLOQ not reported	Cross-reactivity and interference not reported for any of the matrices investigated	Recovery from soil and earthworm tissue and extraction efficiency from maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Shan et al. <sup>96</sup>	Cry1F	Soil	LOD, LLOQ determined empirically for each soil type	Analyzed solutions of soil extracts for matrix effects; Used a 2X dilution to mitigate matrix effects	Mean recoveries ranged 66.9–90.5% depending on soil type; %CV ranged from 5.4% to 13.8%	Used commercial ELISA kit; tested method with 2 analysts across 2 days; %CV = 9.4–14.6%	Performed with a susceptible species; bioassays indicated no protein present; confirmed ELISA results
Daudu et al. <sup>85</sup>	Cry1Ab	Soil, maize tissue	LOD, LLOQ not reported	Cross-reactivity and interference due to matrix components not reported	Recovery from soil and extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Badea et al. <sup>120</sup>	Cry1Ab	Soil, maize tissue	LOD = 0.01 ng/g soil; LLOQ not reported	Analyzed soil free of Cry protein in parallel, no false positives; indicates cross-reactivity not significant; interference not reported	Mean recovery less than 40% in soil; %CV not reported; extraction efficiency from maize tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Miethling-Graff et al. <sup>121</sup>	Cry3Bb1	Soil, maize detritus	LOD = 0.01 ng/g soil; LLOQ not reported	Analyzed soils from non-Bt plots in parallel, no false positives; indicates cross-reactivity not significant; interference not reported	Recovery from soil and extraction efficiency from maize detritus not reported	Used commercial ELISA kit that had been previously validated (Nguyen et al. <sup>131</sup> )	Not performed; unknown if protein detected was fully intact and/or biologically active
Tank et al. <sup>87</sup>	Cry1Ab	Maize detritus, water	LOD = 0.56 ng/mL (maize detritus) and 6 ng/L (water); LLOQ not reported	Used buffer blanks to account of buffer matrix effects; Did not account for matrix effects from maize detritus and water samples	Recovery from water and extraction efficiency from maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Gruber et al. <sup>111</sup>	Cry1Ab	Maize tissue, soil, animal feed, liquid manure	LOD = 0.4 ng/g; Decision Limit and Detection Capacity determined according to European Commission Decision 2002/657/EC34	No cross-reactivity or interference observed in any of the blank matrix samples analyzed	Mean recoveries ranged 68–98% depending on sample matrix; %CV ranged from 4.5 to 16.7% <sup>a</sup>	Intra-assay precision = 5.9% CV; Interassay precision = 14.6% CV	Western blotting used to determine protein fragmentation; ELISA results indicated protein in slurry at 24 weeks, but only fragments observed with Western blots

Table 1. continued

Reference	Protein	Matrix	Sensitivity	Specificity	Accuracy	Precision	Cross Validation
Helassa et al. <sup>122</sup>	Cry1Aa	Soil	LOD = 4 µg/L; LLOQ not reported	No interference due to soil matrix	Mean recoveries ranged 53–66% depending on soil type; %CV ranged from 9.0 to 29.4% <sup>b</sup> depending on soil.	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Shu et al. <sup>132</sup>	Cry1Ab	Earthworm tissue, soil	LOD, LLOQ not reported	Cross-reactivity and interference to soil and earthworm tissue not reported	Recovery from soil and earthworm tissue not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Fejes et al. <sup>69</sup>	Cry4	Water	LOD = 2 ng/mL pure protein, 170–900 ng/mL in samples concentrated via lyophilization; LLOQ not reported	Matrix effects observed in undiluted Bt formulations used in standard curves; matrix effects of field-collected water used in study not reported	Mean recoveries 23–30%; insufficient information to calculate %CV	Used commercial antibodies, but prepared own microplates and antibody-enzyme conjugates; variability between plates, antibody-enzyme batches not reported	Performed with a susceptible species; bioassays indicated increased larval survival as concentration of protein decreased (concentration of protein determined by ELISAs)
Gruber et al. <sup>84</sup>	Cry1Ab	Soil	LOD = 0.4 ng/g soil; Decision Limit (2 ng/g soil) determined according to European Commission Decision 2002/657/EC34	Analyzed soils from non-Bt fields in parallel, no false positives, indicates cross-reactivity not significant; interference not reported	Mean recoveries ranged 49–89% depending on soil type; %CV not reported	Generated ELISA plates in-house; data on variation between or within plates or across days and plate lots not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Wang et al. <sup>124</sup>	Cry1Ab/1Ac fusion protein	Soil, root exudates, water	LOD 0.1 ng/g (soil) and 0.005 ng/mL (water); LLOQ not reported	Analyzed water from non-Bt plots in parallel, no false positives, indicates cross-reactivity not significant; protein detected in soils from non-Bt plots	Mean recoveries ranged 46–82% depending on soil type; %CV not reported; recovery from root exudates not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active
Whiting et al. <sup>123</sup>	Cry1Ab, Cry3Bb1	Soil, sediment, water	LOD = 0.86 ng/g soil, 2.1 ng/L water LLOQ not reported	Previously validated [Muetting et al., <sup>97</sup> Strain et al. <sup>98</sup> ]	Mean recoveries of 77% in soil and sediment, 78% in water; %CV ranged from 9.1% to 17.9% <sup>a</sup>	Previously validated [Muetting et al., <sup>97</sup> Strain et al. <sup>98</sup> ]	Not performed; unknown if protein detected was fully intact and/or biologically active
Xue et al. <sup>125</sup>	Cry3Bb1	Soil, maize detritus	LOD, LLOQ not reported	Analyzed soils from non-Bt plots in parallel, no false positives, indicates cross-reactivity not significant; cross-reactivity and interference in maize detritus not reported	Mean recoveries ranged 10–96% depending on soil type; %CV ranged from 2.5 to 20.7% <sup>a</sup> ; extraction efficiency for maize detritus not reported	Used commercial ELISA kit; additional precision data not reported	Not performed; unknown if protein detected was fully intact and/or biologically active

<sup>a</sup>%CV calculated using mean and standard deviation provided by the authors. <sup>b</sup>%CV calculated using mean, standard error, and sample size provided by the authors.



the growing season and postharvest, with Cry1Ab still detected in one field seven months later.<sup>86</sup> Tank et al.<sup>87</sup> found free Cry protein in 23% of 215 water samples taken from streams near agricultural fields six months after harvest. The stark contrast of these two studies with the previous three studies likely is due to a lack of validation among all the studies. This lack of validation, and the potential consequences, will be discussed further in the “[Cross Validation](#)” section of this paper.

**Advantages and Disadvantages.** The use of ELISAs for detection of Cry proteins in environmental matrices has several advantages and disadvantages. ELISAs are highly sensitive, with detection limits in the ng/mL range, and are highly selective due to the specificity of the antibodies.<sup>77,88</sup> ELISAs are easier to perform and produce results more quickly than conventional methods such as liquid chromatography paired with mass spectrometry (LC/MS). While the amount of time dedicated to sample preparation may be similar, analysis of the samples is much faster with ELISAs. For example, analysis of a 96-well microtiter plate designed to detect Cry1Ab or Cry1Ac protein requires a total analytical time of 3.5–4 h, or 2–2.5 min per sample, while analysis with LC/MS could take 10–15 min per sample, for a total of 16–24 h.<sup>89</sup> The ability to process a large amount of samples in a short time span also makes ELISAs cost-effective.<sup>77</sup> As of May 2015, a 480 well Cry1Ab/1Ac kit from Agdia, Inc. (Elkhart, IN, USA) cost approximately \$600 USD, or \$1.25 per sample (not including controls).<sup>90</sup> Depending on equipment setup, the cost to operate a LC/MS can be \$30–100 per hour. Assuming costs are \$30 per hour, and run time per sample is 10 min, analysis of 480 samples with LC/MS would cost \$2,400 (\$5 per sample).

A drawback with the use of antibodies, however, is that the antibodies may cross-react with closely related proteins, such as antibodies specific for Cry1Ab cross-reacting with Cry1Ac.<sup>88,91,92</sup> Also, nonspecific binding may occur between other proteins in environmental samples and the antibodies, enzymes, or even the plastic microtiter plates utilized in the assays.<sup>93</sup> Further, if the protein structure is altered or the antibody binding sites are damaged, there can be reduced antibody–antigen binding, which may affect the accuracy of the analysis.<sup>77</sup> Conversely, ELISAs can only distinguish between bioactive and nonbioactive proteins when the protein structures are altered significantly. Thus, if only minor structural alterations exist that render the protein inactive against susceptible insect species, but do not affect the binding of the antibodies to the protein epitopes, then a protein that is not bioactive may be detectable by the assay, producing a false-positive result.<sup>70,94</sup> Finally, ELISAs are not readily conducive to multianalyte analysis.<sup>91</sup> Some attempts have been made to allow for detection of multiple analytes, but these are not true multianalyte analyses, as they require dividing a microtiter plate into multiple sections, one for each analyte. This can greatly reduce the number of samples per plate; if samples are screened for three different analytes, the number of samples per plate decreases from 96 to 32.<sup>95</sup>

## ■ VALIDATION OF ELISAS

As with any analytical method, validation of ELISAs is necessary to ensure that the performance of the assay meets specific criteria. These criteria, as well as the scope of the validation procedure, may vary according to the intended use of the assay; however, at a minimum, validation procedures typically address the sensitivity, specificity, accuracy, and precision of ELISAs to ensure that the target analyte can be detected and/or quantified in a reproducible manner. Several excellent articles and book

chapters have been published on the topic of ELISA validation and cover the topic in greater detail than in this review.<sup>71,72,93</sup>

Additionally, several articles have been published describing validated methods for detection of Cry proteins in various matrices.<sup>92,96–99</sup> The remainder of this section will briefly cover the key areas of validation: sensitivity, specificity, accuracy, and precision.

**Sensitivity.** The sensitivity of an ELISA is determined by the smallest amount of target analyte that an assay can reproducibly detect. For a quantitative ELISA, the quantitative range, upper limit of quantitation (ULOQ), and lower limit of quantitation (LLOQ) are important parameters for defining sensitivity. The quantitative range is the range over which the ELISA will produce quantitative results within acceptance criteria; it is determined by the concentrations over which the standard curve produces a linear response. The ULOQ and LLOQ are the highest and lowest concentrations, respectively, that can be measured with an acceptable level of accuracy and precision. They are commonly defined as the highest and lowest points, respectively, on the standard curve. The sensitivity for a qualitative ELISA is often defined by the limit of detection, or the lowest concentration at which it is possible to differentiate between a positive and negative sample. The limit of detection can be defined as an absorbance reading that is two or three standard deviations above a negative or background control sample.<sup>72,93</sup>

**Specificity.** The specificity of an ELISA is the capacity of the assay to differentiate between the Cry protein of interest and other components that may be present in the samples. There are two main constituents to specificity: cross-reactivity or interference of other transgenic proteins, and cross-reactivity or interference of matrix components. Checking for cross-reactivity with other proteins (including transgenic proteins) is essential to determining if the antibodies utilized in the ELISA will bind to other closely related proteins, such as Cry1Ab and Cry1Ac. Fortunately, for researchers using commercial ELISA kits, this work is typically performed by the manufacturer. Equally important is determining if the matrix (e.g., soil, tissue, water) can affect the capacity to detect and/or quantify the Cry protein of interest. Matrix components may contain homologous endogenous counterparts that could potentially cross-react with the antibodies, producing a response. Nonspecific binding between the antibodies and matrix components also may produce a positive response, while decreased ability of the assay to quantify the protein may occur if matrix components interfere with the antibody–Cry protein interaction.<sup>93</sup>

**Accuracy.** The accuracy of an ELISA is its ability to determine the true amount of Cry protein in a sample. The accuracy of an ELISA can be determined through the use of extraction efficiency and fortification-and-recovery studies. Extraction efficiency is used to express the capability of an extraction method to separate the Cry protein of interest from the sample matrix, which is determined by utilizing serial extractions of the same sample. The amount of protein in the first extraction is divided by the sum of the protein in all the extractions. Extraction efficiencies between 70% and 100% with a coefficient of variation (%CV) of less than 20% are ideal. Fortification-and-recovery testing (also known as spike-and-recovery) is used to determine recovery across multiple points in the quantitative range. In this test, negative soil, tissue, or water samples (samples free of Cry proteins) are fortified or spiked with a known amount of protein. The samples are then extracted once according to the extraction procedure, and the

amount of protein recovered is divided by the total amount of protein initially added. Ideal mean recovery values are between 70% and 120%, with a CV of less than 20%.<sup>72,93</sup> The inherent variability in biological systems and the small quantities of Cry proteins that are typically found in environmental samples make accurate measurements challenging and can lead to recovery values greater or less than 100%. Some major factors in the extraction process that can affect the recovery of Cry proteins in the extraction efficiency and fortification-and-recovery procedures from various environmental matrices include the type of extractant/solvent used, number of times a sample is extracted and the duration of each extraction, and the type of agitation used (grinding, shaking, etc.).

**Precision.** The precision, or reproducibility, of an ELISA describes the amount of variation that may occur within an assay or across multiple assays. The use of commercial ELISA kits can help reduce some of this variability, especially if kits from the same lot number (i.e., same source of antibodies, enzyme conjugate, etc.) are used for all samples. However, not all sources of variability can be accounted for by the use of commercial kits, such as day-to-day and analyst-to-analyst variability, and thus, the precision of the assay needs to be verified. Assay precision can be tested by analyzing aliquots of the same quality control samples of known concentration multiple times (across days, analysts, etc.). The mean and standard deviation of all of these samples can then be used to calculate the %CV for the samples. Ideally, the %CV will be less than 20%, though this can vary depending on the intended use of the assay.<sup>71,72,93</sup>

## CROSS VALIDATION

The procedures and criteria for validating the analytical performance of ELISAs are well described, as previously discussed. One area that is not well-defined is the cross validation of ELISA results. False-positive ELISA results, a positive detection when no antigen is present, are a known issue with ELISAs across many areas of science.<sup>100–102</sup> One potential source of false-positive results when analyzing environmental samples for Cry proteins is the detection of partially degraded proteins by the ELISA. This may result from the prevalent use of polyclonal antibodies in commercial ELISA kits. Polyclonal antibodies bind to multiple epitopes on a protein; therefore, a partially degraded protein may have a sufficient number of antibody binding sites still intact to allow for a positive detection.

Several published studies support this possibility. Einspanier et al.<sup>103</sup> collected samples from the gastrointestinal tract (GIT) at slaughter of cows fed either transgenic Cry1Ab maize or nontransgenic isoline maize; the samples were analyzed with a commercial ELISA kit for Cry1Ab/1Ac. The results indicated that Cry1Ab protein resisted digestion and appeared to accumulate in some intestinal juice samples. Cross-reactivity of the ELISA with animal, microbial, or plant compounds was ruled out because this phenomenon was not observed in cows fed nontransgenic isoline maize. In a follow-up study, the authors hypothesized that the positive ELISA detection may have been the result of a fragmented, yet immunoreactive Cry1Ab protein reacting with the antibodies.<sup>101</sup> This hypothesis was tested in a second feeding study. Cows were fed either transgenic Cry1Ab maize or nontransgenic isoline maize, and GIT samples were collected at slaughter. In addition to ELISAs, Western blotting was performed on all samples. The ELISA results emulated the initial study; Cry1Ab protein was detected in all samples, and the concentration appeared to increase

during passage through the GIT. Cross-reactivity with animal, microbial, or plant components was again ruled out. The Western blot data told a different story. Fully intact Cry1Ab was not detected in any GIT samples; fragment bands at approximately 17 and 34 kDa were observed in cows fed transgenic maize, while no comparable protein bands were observed in cows fed nontransgenic maize. The results support the hypothesis that the Cry1Ab protein was fragmented, yet still capable of immunoreacting with the ELISA antibodies.<sup>101</sup>

Similar results were observed in earthworm tissue samples. Emmerling et al.<sup>104</sup> collected samples of earthworm casts and gut content and analyzed the samples for the presence of Cry1Ab with ELISAs and Western blotting. ELISA results indicated a decreasing, yet still detectable concentration of protein as the protein moved through the earthworms' digestive tract. However, no fully intact protein was observed in any of the samples. Three fragments with an approximate size of 17, 23, and 31 kDa were detected in the foregut and midgut samples. Western blotting did not indicate that fragments were present in samples of the hindgut or cast material, even though Cry1Ab was detected in these samples by ELISA. The authors did not specify if blank control samples were performed; thus, positive ELISA detections in the hindgut and cast samples may be the result of cross-reactivity with animal, microbial, plant, or soil components.<sup>104</sup>

These studies provide examples of why validation is necessary to ensure that only fully intact Cry proteins are being detected by ELISAs. Detection of nonbioactive Cry protein fragments by ELISAs may lead to an overestimation of the amount of protein in the environment. These overestimations could potentially have impacts on the risk assessments for transgenic crops and insecticide formulations containing Cry proteins. Although validating ELISA results with bioassays is ideal, Western blotting and LC/MS also can be used to validate results generated by ELISAs.

**Bioassays.** The best way to determine if biological activity of the Cry proteins remains is to perform bioassays on environmental samples with an insect species susceptible to the protein of interest. There are many different bioassay methods, depending on the sample matrix to be studied. Soil samples may be laid over the top of the prepared insect diet or incorporated directly into the diet.<sup>96,105</sup> Alternatively, Cry proteins may be extracted from soil samples, and then the extract can be incorporated into the diet or overlaid on top of the diet.<sup>106</sup> Plant tissue and detritus can be analyzed by direct feeding on the tissue, or by incorporating the tissue or detritus into the diet.<sup>107,108</sup> Water samples may be analyzed by placing the insects directly into the water.<sup>69</sup> After a predetermined incubation period on the sample-infused diet, insect mortality and other parameters, such as insect weight or head capsule width, may be recorded to determine lethal and sublethal effects. Bioassays are only semiquantitative; exact protein concentrations are impossible to define, but based on known LC<sub>50</sub> and EC<sub>50</sub> values (the concentration needed to cause mortality or a specific effect in 50% of the sample population, respectively), a general idea of the concentrations present can be determined. Other drawbacks that limit the usefulness of bioassays for cross validation are that they are time-consuming, expensive, and labor-intensive to set up and analyze, along with maintaining insect colonies for further studies. Also, results may be skewed if an increase in sample material (soil, plant tissue) in the diet is needed to increase protein concentrations to levels sufficient to cause negative effects. This increase in sample material may

cause a decrease in the essential nutrients needed for insect survival, which could result in higher than anticipated mortality or effect levels. Thus, bioassay results also should be validated.

The three studies discussed below give a sampling of how bioassays have been used for cross validation. Head et al.<sup>109</sup> analyzed soil samples for the presence of Cry1Ac from cotton with ELISAs and bioassays. Bioassays were performed by mixing soil with water to form a slurry, which was then mixed with an agar-based diet. After the diet solidified, one first-instar tobacco budworm larva (*Heliothis virescens*) was introduced into each well. Larval survival and insect weights were determined after 7 days. The bioassays supported the ELISA results that indicated no protein was present in any of the soil samples.<sup>109</sup>

Shan et al.<sup>96</sup> analyzed soil samples for the presence of Cry1F from maize with ELISAs and bioassays. Rhizosphere soil samples were collected and diluted by a factor of 10 (weight/volume) with agar. This suspension was then laid over the top of the previously prepared insect diet, and one neonate *H. virescens* was placed in each well. Mortality and insect weights were recorded after 6 days. Cry1F was not detected in any of the rhizosphere soil samples by bioassays, which corroborated the ELISA results.<sup>96</sup>

Zwahlen et al.<sup>108</sup> analyzed maize detritus collected from litter bags over a period of several months for the presence of Cry1Ab with ELISAs and bioassays. In the first year of the study, maize detritus was incorporated directly into the insect diet and fed to neonate European corn borers (*Ostrinia nubilalis*); percentage mortality and weights of surviving insects were recorded after 6 days. In the second year, maize detritus was mixed with extraction buffer and then added to the insect diet; mortality and insect weights were recorded after 5 days. In both years, larval mortality decreased over time. This finding supported the ELISA results, which showed that the Cry1Ab concentration in maize detritus also decreased over time.<sup>108</sup>

**Western Blotting.** Western blotting is a technique used to identify specific proteins in a sample. First, gel electrophoresis is used to separate proteins and fragments in a sample by size. The proteins and fragments are transferred, or electroblotted, onto a nitrocellulose or polyvinylidene difluoride membrane. Antibodies specific to the protein of interest are incubated with the membrane and bind to the protein. Finally, a substrate is added which allows for visualization of the protein bands on the membrane. Depending on the type of enzyme conjugated to the antibody and the type of substrate used, detection can be colorimetric, chemiluminescent, radioactive, or fluorescent.<sup>110</sup> Western blotting is capable of detecting small quantities of protein in a sample. However, Western blotting is still time-consuming, expensive, semiquantitative and has a reduced throughput. These drawbacks are likely a few of the reasons Western blotting is not used more often.

In addition to the studies described above, two other studies also have used Western blotting to validate their results. Gruber et al.<sup>111</sup> traced the fate of Cry1Ab protein in transgenic maize, through animal feed, and into liquid manure. Cry1Ab was detected by ELISA in transgenic maize and animal feed prepared from transgenic maize; Western blotting confirmed the presence of fully intact protein. In the liquid manure samples, ELISAs showed a decrease in Cry1Ab concentration over time, which was confirmed by Western blotting; however, after 24 weeks of storage, ELISAs still showed that Cry1Ab was present, while Western blotting detected only a 34 kDa fragment.<sup>111</sup>

Paul et al.<sup>112</sup> analyzed GIT samples from cows fed transgenic Cry1Ab maize or nontransgenic isolate maize. The ELISA results echoed the Einspanier et al.<sup>103</sup> and Lutz et al.<sup>101</sup> studies; Cry1Ab appeared to accumulate as it moved through the GIT. Western blotting determined that small quantities of fully intact Cry1Ab were present in all samples, but that the accumulation of protein observed in the ELISA results could be attributed to an increase in fragments approximately 17, 34, and 42 kDa in size, and not due to an increase in fully intact Cry1Ab.<sup>112</sup>

**LC/MS.** Liquid chromatography paired with mass spectrometry (LC/MS) is another analytical method that may be used to detect Cry proteins in environmental samples. In this method, proteins are digested into peptides via proteolytic enzymes. These peptides are separated by high efficiency nanocolumn liquid chromatography, which feeds the peptides directly into the mass spectrometer. The peptides are ionized by the mass spectrometer, and all intact peptide ions are measured. The instrument then selects peptide ions based on predetermined criteria such as charge state or mass-to-charge ratio ( $m/z$ ) and subjects these selected peptides to collisionally induced dissociation (CID), which causes the peptide ions to fragment in a predictable manner. The CID fragmentation pattern can be used to determine the sequence of the peptide; this sequence can be compared to databases containing sequences of known proteins to identify the protein in the sample and determine if it is fully intact or a fragment. This method allows for identification of a single protein or fragment in a complex mixture of proteins without the need for further purification.<sup>113</sup>

Currently, the study of Cry proteins with LC/MS has been limited to identification of new toxins and investigations into the mode of action of Cry proteins. Yang et al.<sup>114</sup> utilized two-dimensional liquid chromatography–tandem mass spectrometry to analyze protein samples from *Bacillus thuringiensis* strain 4.0718. The authors identified more than 1,000 unique proteins; 11 of these proteins were determined to be insecticidal Cry proteins. Bayyareddy et al.<sup>115</sup> used LC/MS to identify aminopeptidases and alkaline phosphatases, which are known receptors for Cry4Ba, in the detergent-resistant membranes (also known as lipid rafts) of the yellow fever mosquito (*Aedes aegypti*). These detergent-resistant membranes had previously been suggested as potential entry points for bacterial pathogens and their toxins. No studies using LC/MS to analyze environmental samples or validate ELISA results were found in this current literature review. This is not surprising, as the high initial setup costs, maintenance costs, specialized training required to operate the instrument, and long sample analysis time (>90 min<sup>114,115</sup>) make LC/MS a less favorable option for detection of Cry proteins and validation of ELISA results in environmental samples.

Hu and Owens<sup>116</sup> demonstrated proof of concept for the detection of Cry proteins with LC/MS. They analyzed transgenic maize tissue extracts with LC/MS for the presence of three foreign proteins and were able to determine the concentration of all three proteins in a single sample extract. Further, when compared to ELISAs, the LC/MS method had less day-to-day variation and produced comparable results; most of the LC/MS data were within two standard deviations of the ELISA data.<sup>116</sup>

## ■ CRITICAL REVIEW OF PUBLISHED STUDIES

The final section of this article reviews several published studies and critiques their usage of ELISAs for detection of Cry proteins in the environment. This is not meant to be an exhaustive



review of all published articles, but rather is intended to provide the reader with a general assessment of how the scientific community is utilizing ELISAs for Cry protein detection and quantification and identify areas for improvement. Table 1 summarizes the reviewed articles.

**Sensitivity.** The sensitivity of ELISAs is one of the areas of validation that is most commonly reported in research articles. The limit of detection (LOD) is most often reported, while the lower limit of quantitation (LLOQ) is reported less often. A few studies reported decision limits instead of LLOQ; these decision limits were determined according to governmental criteria.<sup>84,111,117</sup> Additionally, a few studies reported only the LOD for the commercial ELISA kit used in the study, and did not report the LOD or LLOQ for the different matrices investigated.<sup>118,119</sup> Of the studies that reported LOD values, the results confirm that ELISAs are a sensitive method of detection; the LOD in these studies ranged from 0.01 ng/g (0.01 ppb) to 4 µg/g (4 ppb) in soil and 2.1 ng/L (2.1 ppt) to 6 µg/L (6 ppb) in water.<sup>87,120–123</sup>

One-fourth (nine out of 26) of the studies reviewed failed to report any validation of sensitivity; thus, the sensitivity of the recovery methods and ELISA procedures used in these studies is unknown. In at least one study, the authors reported that no Cry1Ab protein was detected in soil that was in contact with decaying transgenic maize tissue.<sup>85</sup> Since the authors did not report any sensitivity data, it is impossible to determine if, in fact, no protein was present, or if Cry1Ab protein was present in the soil, but the recovery methods and ELISA procedures used in the study were not sensitive enough to detect the protein.

**Specificity.** The use of commercial ELISA kits targeted for detection of only one or two proteins has helped resolve some of the specificity issues that occur in environmental fate studies, especially when multiple Cry proteins may be present. However, cross-reactivity of the antibodies with components in the sample matrix or interference of the sample matrix, preventing antibodies from binding to Cry proteins, still needs to be addressed, regardless of whether or not a commercial kit is used. For example, Shan et al.<sup>96</sup> used a commercial ELISA kit and observed slight matrix effects in samples of soil extracts. To mitigate the matrix effects, a 2× dilution was used for all samples.

Several studies (14 of 26) analyzed blank soil or water samples for the presence of Cry proteins in parallel with their analysis on environmental samples. In nearly all of those control samples, no positive detections occurred; thus, cross-reactivity with sample matrix components was excluded. However, these blank samples do not account for the ability of matrix components to interfere with the quantitation of a protein. Testing for interference (as well as cross-reactivity) should be performed by mixing a 2× standard curve with the blank sample matrix (water, soil extract, etc.), resulting in a 1× standard curve in a 2× dilution of the matrix. Using a 1× standard curve prepared in assay buffer as a reference, the differences between the theoretical and observed values for the points in the standard curve prepared in the matrix can be calculated. Differences of greater than 20% may indicate interference or cross-reactivity, though this may vary.<sup>93</sup> Another problem with running only blank matrix samples can be observed in the study by Wang et al.<sup>124</sup> In that study, the authors detected protein in soil samples from plots planted with non-Bt rice. Since no further sensitivity validation steps were performed, it is difficult to determine if the positive detection is the result of a basal level

of protein in the soil, as the authors suggest, or is due to cross-reactivity to soil components.<sup>124</sup>

As with sensitivity, approximately one-fourth (9 out of 26) of the studies reviewed failed to report any validation of specificity. Therefore, the extent of the effect, if any, that cross-reactivity and interference of other proteins and matrix components may have on the results of these studies is difficult to ascertain.

**Accuracy.** Approximately one-third (10 out of 26) of the studies reviewed failed to report any recovery or extraction efficiency values for accuracy. Thus, it becomes difficult to determine how efficient these extraction methods were at recovering Cry proteins from various environmental matrices. Further, of the studies that do include recovery values, many of the recovery values are significantly below the acceptable recovery range of 70–120%. Recovery values of 10–50% in soil are common, although higher recoveries are attainable.<sup>86,111,123,124</sup> Soil type has a significant impact on the recovery values of Cry proteins observed in soil samples. Soils high in clay and silt content typically yield poor recovery of Cry proteins (10–50%) while soils high in sand content yield better recoveries (75–98%).<sup>84,96,125</sup> Recovery of Cry proteins in water also is highly variable, ranging from 23% to 78%.<sup>69,123</sup> One study avoided this issue by analyzing the water directly; however, since no protein was detected in the water samples, extracting the samples (i.e., concentrating) would have been advisable to increase the sensitivity of the method.<sup>61</sup>

Low recovery values are a known problem with extraction of Cry proteins from environmental matrices.<sup>64</sup> One mechanism to manage this issue is to perform spike and recovery on multiple samples and determine the variation between the samples. A % CV of less than 20% provides support that a researcher is using an accurate extraction method, even though recovery of the protein may be less than 70%.<sup>93</sup> Only one of the studies reviewed provided %CV values; Shan et al.<sup>96</sup> reported %CV values of 5.4–13.8% for soil recovery. An additional four studies reported recovery means and standard deviations.<sup>109,111,123,125</sup> Thus, the % CV for these studies can be calculated by dividing the standard deviation by the mean and multiplying by 100%. In these studies, the calculated %CV values range from 4.7% to 21.2% depending on the sample matrix. Three additional studies reported means, standard error, and sample size.<sup>82,122,126</sup> Standard deviation in these studies can be calculated through the equation: standard error =  $\frac{\text{standard deviation}}{\sqrt{\text{sample size}}}$ , or written another

way, standard deviation = standard error ×  $\sqrt{\text{sample size}}$ . In these three studies, the calculated %CV values ranged from 6.1% to 35.1%. Eight of the reviewed studies provided the standard error or percentage recovery, but did not provide enough additional information (i.e., sample size) to calculate %CV for the studies. As noted above, the remainder of the studies failed to report percentage recovery.

**Precision.** Precision is the area of validation reported the least in the literature reviewed here. Only 6 of 26 studies reported any information on validation of assay precision. Two studies used assay methods that previously had been validated for precision.<sup>121,123</sup> Shan et al.<sup>96</sup> investigated the precision of the assay across analysts and days (%CV = 9.4–14.6%), while Gruber et al.<sup>111</sup> validated the intra- and interassay precision (%CV = 5.9% and 14.6%, respectively). The final two studies reanalyzed plates that had a %CV greater than 10%.<sup>61,82</sup>

Three-fourths (20 of 26) of the studies not reporting precision data did utilize commercial ELISA kits. Use of commercial kits is

advantageous, as these kits undergo rigorous testing by the manufacturer during development to ensure uniformity with plates, between plates, across antibody lots, etc. Thus, some of the concerns regarding assay precision can be mitigated by the use of a commercial ELISA kit, while other areas still need to be validated (e.g., variation between analysts, etc.). Finally, five studies prepared ELISA plates in their laboratories using lab-generated or commercial sources of antibodies.<sup>69,84,108,109,118</sup>

All of these studies failed to report precision data. This is significant, as the processes for preparing these plates in research laboratories is most likely not as refined as the processes used in commercial manufacturing facilities, which could result in inconsistencies across plates or even within a single plate. Additionally, there may be significant variability between lots of lab-generated antibodies. All of these factors can affect the variability of the assays, making comparisons between samples on separate plates difficult.

**Cross Validation.** A vast majority of the studies failed to perform any form of cross validation. Thus, it is impossible to determine if the Cry proteins detected in environmental samples were fully intact and/or biologically active. A few papers recognized that the protein detected may not be fully intact and/or biologically active. Baumgarte and Tebbe<sup>86</sup> recognized that they could not “claim that the immunoreactive Cry1Ab protein detected in soils and plant residues was actually biologically active.” Nguyen and Jehle<sup>127</sup> also acknowledged that “it is not clear whether these ELISA detectable Cry1Ab residues still retain their bioactivity.” Gruber et al.<sup>117</sup> did not perform cross validation, but protein was not detected in any of the soil samples, rendering cross validation unnecessary.

Seven papers performed bioassays with an insect species that was susceptible to the protein of interest in the study. In five of the studies, the bioassay results confirmed the ELISA results. In the studies by Zwahlen et al.<sup>108</sup> and Fejes et al.,<sup>69</sup> the bioassay results showed decreasing mortality and sublethal effects, as the protein levels, as determined by ELISA, also decreased. Bioassays performed by Head et al.<sup>109</sup> and Shan et al.<sup>96</sup> indicated that no Cry protein was present in any of the samples, which corresponded to the ELISA results. Wang et al.<sup>126</sup> performed bioassays on soil samples from the rhizosphere region of transgenic rice plants and observed no significant effects, confirming the ELISA results; however, the authors did not perform bioassays on samples taken from soil amended with transgenic rice tissue.

The remaining two studies produced the most interesting results. Bioassays performed by Marchetti et al.<sup>128</sup> suggested toxicity of the proteins decreased more rapidly than estimated by ELISA results. This is interesting because it indicates that the ELISAs may be detecting nonbioactive forms of the protein. Gruber et al.<sup>111</sup> cross validated their results with Western blotting instead of bioassays. The results showed that Cry1Ab fragments of 17, 34, and 42 kDa, as well as the full-length 65 kDa parent molecule, were detected in transgenic plant tissue and animal feed from transgenic plants. Only the 34 kDa fragment and the full-length protein were detected in liquid manure. The most interesting result, however, is that, after 24 weeks of storage, the full-length protein had been degraded and only the 34 kDa fragment remained, yet Cry1Ab could still be detected in the slurry by ELISA at a concentration of approximately 1 µg/g. This is interesting because, as with the Marchetti et al.<sup>128</sup> study, it indicates that the ELISA may be detecting degraded, nonbioactive forms of the protein.

ELISAs have emerged as the predominant method for detecting Cry proteins in the environment. ELISA validation methods are necessary to ensure usable data are generated in environmental fate studies. These methods, and their associated acceptance criteria, have been validated through years of research, but they have yet to be widely adopted by environmental fate researchers. More than 90% of the studies reviewed failed to perform one or more of the five key areas of validation. Thus, substantial improvement in estimating the concentrations of Cry proteins in environmental samples can be made by simply increasing the practice of utilizing validation methods. Additionally, little to no information exists on whether Cry protein fragments can be detected by ELISAs. Research is needed to determine if Cry protein fragments can be detected by ELISAs and whether the fragments retain biological activity. Understanding the fate of Cry protein fragments can help determine if a need exists for cross validation of ELISA results with another analytical technique. Increasing the accuracy of environmental measurements will increase the usefulness of these data to regulators and will allow for a more efficient regulatory process.

## ■ ASSOCIATED CONTENT

### § Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b03766.

Table listing U.S. manufacturers of ELISA kits for detection of Cry proteins (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*(J.R.C.) Phone: (515) 294-4776. Fax: (515) 294-7406. E-mail: jcoats@iastate.edu.

### Notes

The authors declare no competing financial interest.

### Disclosure

Mention of a proprietary product does not constitute an endorsement or recommendation by Iowa State University or USDA for its use.

## ■ REFERENCES

- (1) Ishiwata, S. On a kind of severe flacherie (sotto disease). *Dainihon Sanshi Kaiho* **1901**, 114, 1–5.
- (2) Beegle, C. C.; Yamamoto, T. Invitation paper (C.P. Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development. *Can. Entomol.* **1992**, 124, 587–616.
- (3) Ishiwata, S. About “sottokin,” a bacillus of a disease of the silkworm. *Dainihon Sanshi Kaiho* **1905**, 161, 1–5.
- (4) Angus, T. A. A Bacterial Toxin paralysing Silkworm Larvae. *Nature* **1954**, 173, 545–546.
- (5) Pigott, C. R.; Ellar, D. J. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.* **2007**, 71, 255–281.
- (6) Soberón, M.; Gill, S. S.; Bravo, A. Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cell. Mol. Life Sci.* **2009**, 66, 1337–1349.
- (7) Jurat-Fuentes, J. L.; Adang, M. J. Cry toxin mode of action in susceptible and resistant *Heliothis virescens* larvae. *J. Invertebr. Pathol.* **2006**, 92, 166–171.
- (8) Ujvary, I. Pest control agents from natural products. In *Hayes' Handbook of Pesticide Toxicology*, 3rd ed.; Krieger, R., Ed.; Academic Press: 2010; Vol. 1, pp 119–230.
- (9) Schnepf, E.; Crickmore, N.; Van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J.; Zeigler, D. R.; Dean, D. H. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **1998**, 62, 775–806.



- (10) Lambert, B.; Peferoen, M. Insecticidal promise of *Bacillus thuringiensis*. *BioScience* **1992**, *42* (2), 112–122.
- (11) de Maagd, R. A.; Bosch, D.; Stiekema, W. *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends Plant Sci.* **1999**, *4* (1), 9–13.
- (12) Glare, T. R.; O'Callaghan, M. *Bacillus thuringiensis: biology, ecology and safety*; Wiley: Chichester, 2000; Vol. 350.
- (13) Navon, A. *Bacillus thuringiensis* application in agriculture. In *Entomopathogenic bacteria: from laboratory to field application*, Charles, J.-F., Delecluse, A., Nielsen-LeRoux, C., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp 355–369.
- (14) Teakle, R. E. Present use of, and problems with, *Bacillus thuringiensis* in Australia. *Agric., Ecosyst. Environ.* **1994**, *49*, 39–44.
- (15) Gindin, G.; Navon, A.; Saphir, N.; Protasov, A.; Mendel, Z. Differing susceptibility to *Bacillus thuringiensis* formulations of *Thaumatococcus wilkinsoni* populations between forests with different Bt management in Israel. *Phytoparasitica* **2007**, *35*, 179–190.
- (16) Zuckerman, B. M.; Dicklow, M. B.; Acosta, N. A strain of *Bacillus thuringiensis* for the control of plant-parasitic nematodes. *Biocontrol Sci. Technol.* **1993**, *3*, 41–46.
- (17) Culliney, T. W.; Grace, J. K. Prospects for the biological control of subterranean termites (Isoptera: Rhinotermitidae), with special reference to *Coptotermes formosanus*. *Bull. Entomol. Res.* **2000**, *90*, 9–21.
- (18) Regis, L.; Silva-Filha, M. H.; Nielsen-LeRoux, C.; Charles, J.-F. Bacteriological larvicides of dipteran disease vectors. *Trends Parasitol.* **2001**, *17*, 377–380.
- (19) Araújo-Coutinho, C. J. P. C. Biological control program against simuliids in the State of São Paulo, Brazil. *Memórias do Instituto Oswaldo Cruz* **1995**, *90*, 131–133.
- (20) Gray, E.; Adler, P.; Coscaron-Arias, C.; Coscaron, S.; Noblet, R. Development of the first black fly (Diptera: Simuliidae) management program in Argentina and comparison with other programs. *J. Am. Mosq. Control Assoc.* **1999**, *15*, 400–406.
- (21) Dubois, N. R.; Lewis, F. B. What is *Bacillus thuringiensis*. *J. Arboric.* **1981**, *7* (9), 233–240.
- (22) Fischhoff, D. A.; Bowdish, K. S.; Perlak, F. J.; Marrone, P. G.; McCormick, S. M.; Niedermeyer, J. G.; Dean, D. A.; Kusano-Kretzmer, K.; Mayer, E. J.; Rochester, D. E.; Rogers, S. G.; Fraley, R. T. Insect Tolerant Transgenic Tomato Plants. *Bio/Technology* **1987**, *5*, 807–813.
- (23) Vaecq, M.; Reynaerts, A.; Hofte, H.; Jansens, S.; De Beuckeleer, M.; Dean, C.; Zabeau, M.; Montagu, M. V.; Leemans, J. Transgenic plants protected from insect attack. *Nature* **1987**, *328*, 33–37.
- (24) Barton, K. A.; Whiteley, H.; Yang, N.-S. *Bacillus thuringiensis*  $\delta$ -Endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiol.* **1987**, *85*, 1103–1109.
- (25) Perlak, F. J.; Deaton, R. W.; Armstrong, T. A.; Fuchs, R. L.; Sims, S. R.; Greenplate, J. T.; Fischhoff, D. A. Insect Resistant Cotton Plants. *Bio/Technology* **1990**, *8*, 939–943.
- (26) Fujimoto, H.; Itoh, K.; Yamamoto, M.; Kyoizuka, J.; Shimamoto, K. Insect resistant rice generated by introduction of a modified  $\delta$ -endotoxin gene of *Bacillus thuringiensis*. *Bio/Technology* **1993**, *11*, 1151–1155.
- (27) Perlak, F. J.; Stone, T. B.; Muskopf, Y. M.; Petersen, L. J.; Parker, G. B.; McPherson, S. A.; Wyman, J.; Love, S.; Reed, G.; Biever, D. Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant Mol. Biol.* **1993**, *22*, 313–321.
- (28) Kozziel, M. G.; Beland, G. L.; Bowman, C.; Carozzi, N. B.; Crenshaw, R.; Crossland, L.; Dawson, J.; Desai, N.; Hill, M.; Kadwell, S.; Launis, K.; Lewis, K.; Maddox, D.; McPherson, K.; Meghji, M. R.; Merlin, E.; Rhodes, R.; Warren, G. W.; Wright, M.; Evola, S. V. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Technology* **1993**, *11*, 194–200.
- (29) James, C.; Krattiger, A. F. Global review of the field testing and commercialization of transgenic plants: 1986 to 1995. ISAAA Briefs 1–1996, 1996.
- (30) Fernandez-Cornejo, J.; Wechsler, S.; Livingston, M.; Mitchell, L. *Genetically Engineered Crops in the United States*. U.S. Department of Agriculture, Economic Research Service, 2014.
- (31) Acreage. U.S. Department of Agriculture, National Agricultural Statistics Service, Washington, DC, 2015.
- (32) Kilman, S. Monsanto Co. shelves seed that turned out to be a dud of a spud. *Wall Street Journal*, Mar 21, 2001.
- (33) Ming High, S.; Cohen, M. B.; Yao Shu, Q.; Altosaar, I. Achieving successful deployment of Bt rice. *Trends Plant Sci.* **2004**, *9*, 286–292.
- (34) McPherson, R. M.; MacRae, T. C. Evaluation of transgenic soybean exhibiting high expression of a synthetic *Bacillus thuringiensis* cryIA transgene for suppressing lepidopteran population densities and crop injury. *J. Econ. Entomol.* **2009**, *102*, 1640–1648.
- (35) Yu, H.; Li, Y.; Li, X.; Romeis, J.; Wu, K. Expression of CryIAc in transgenic Bt soybean lines and their efficiency in controlling lepidopteran pests. *Pest Manage. Sci.* **2013**, *69*, 1326–1333.
- (36) Bernardi, O.; Malvestiti, G. S.; Dourado, P. M.; Oliveira, W. S.; Martinelli, S.; Berger, G. U.; Head, G. P.; Omoto, C. Assessment of the high-dose concept and level of control provided by MON 87701  $\times$  MON 89788 soybean against *Anticarsia gemmatilis* and *Pseudoplusia includens* (Lepidoptera: Noctuidae) in Brazil. *Pest Manage. Sci.* **2012**, *68*, 1083–1091.
- (37) Shelton, A. M. Genetically engineered vegetables expressing proteins from *Bacillus thuringiensis* for insect resistance. *GM Crops & Food* **2012**, *3*, 175–183.
- (38) Fleisher, S. J.; Hutchison, W. D.; Naranjo, S. E. Sustainable management of insect-resistant crops. In *Plant Biotechnology: Experience and Future Prospects*; Riccio, A., Chopra, S., Fleisher, S., Eds.; Springer International Publishing: Switzerland, 2014; pp 115–127.
- (39) Shelton, A. The long road to commercialization of Bt brinjal (eggplant) in India. *Crop Prot.* **2010**, *29*, 412–414.
- (40) Cultivation of Bt brinjal begins. *The Daily Star*, Jan 22, 2014, <http://www.thedailystar.net/cultivation-of-bt-brinjal-begins-7971> (accessed May 4, 2015).
- (41) Stewart, C. N., Jr.; Adang, M. J.; All, J. N.; Raymer, P. L.; Ramachandran, S.; Parrott, W. A. Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis* cryIAc gene. *Plant Physiol.* **1996**, *112*, 115–120.
- (42) Cao, J.; Brants, A.; Earle, E. D. Cauliflower plants expressing a cryIC transgene control larvae of diamondback moths resistant or susceptible to CryIA, and cabbage loopers. *J. New Seeds* **2003**, *5*, 193–207.
- (43) Cao, J.; Zhao, J. Z.; Tang, J.; Shelton, A.; Earle, E. Broccoli plants with pyramided cryIAc and cryIC Bt genes control diamondback moths resistant to CryIA and CryIC proteins. *Theor. Appl. Genet.* **2002**, *105*, 258–264.
- (44) Paul, A.; Sharma, S.; Sresty, T.; Devi, S.; Bala, S.; Kumar, P.; Saradhi, P. P.; Frutos, R.; Altosaar, I.; Kumar, P. A. Transgenic cabbage (*Brassica oleracea* var. capitata) resistant to Diamondback moth (*Plutella xylostella*). *Indian J. Biotechnol.* **2005**, *4*, 72–77.
- (45) Li, X.-b.; Mao, H.-Z.; Bai, Y.-Y. Transgenic plants of rutabaga (*Brassica napobrassica*) tolerant to pest insects. *Plant Cell Rep.* **1995**, *15*, 97–101.
- (46) Rao, H.; Wu, N.; Huang, M.; Fan, Y.; Wang, M. Two insect-resistant genes were transferred into poplar hybrid and transgenic poplar show insect-resistance. *Prog. Biotechnol.* **2001**, *18*, 239–246.
- (47) Génissel, A.; Augustin, S.; Courtin, C.; Pilate, G.; Lorme, P.; Bourguet, D. Initial frequency of alleles conferring resistance to *Bacillus thuringiensis* poplar in a field population of *Chrysomela tremulae*. *Proc. R. Soc. London, Ser. B* **2003**, *270*, 791–797.
- (48) Harcourt, R. L.; Kyoizuka, J.; Floyd, R. B.; Bateman, K. S.; Tanaka, H.; Decroocq, V.; Llewellyn, D. J.; Zhu, X.; Peacock, W. J.; Dennis, E. S. Insect- and herbicide-resistant transgenic eucalypts. *Mol. Breed.* **2000**, *6*, 307–315.
- (49) Losey, J. E.; Rayor, L. S.; Carter, M. E. Transgenic pollen harms monarch larvae. *Nature* **1999**, *399*, 214–214.

- (50) Jesse, L. C. H.; Obrycki, J. J. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia* **2000**, *125*, 241–248.
- (51) Hellmich, R. L.; Siegfried, B. D.; Sears, M. K.; Stanley-Horn, D. E.; Daniels, M. J.; Mattila, H. R.; Spencer, T.; Bidne, K. G.; Lewis, L. C. Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 11925–11930.
- (52) Anderson, P. L.; Hellmich, R. L.; Sears, M. K.; Sumerford, D. V.; Lewis, L. C. Effects of Cry1Ab-expressing corn anthers on monarch butterfly larvae. *Environ. Entomol.* **2004**, *33*, 1109–1115.
- (53) Dively, G. P.; Rose, R.; Sears, M. K.; Hellmich, R. L.; Stanley-Horn, D. E.; Calvin, D. D.; Russo, J. M.; Anderson, P. L. Effects on monarch butterfly larvae (Lepidoptera: Danaidae) after continuous exposure to Cry1Ab-expressing corn during anthesis. *Environ. Entomol.* **2004**, *33*, 1116–1125.
- (54) Sears, M. K.; Hellmich, R. L.; Stanley-Horn, D. E.; Oberhauser, K. S.; Pleasants, J. H.; Mattila, H. R.; Siegfried, B. D.; Dively, G. P. Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 11937–11942.
- (55) Shirai, Y.; Takahashi, M. Effects of transgenic Bt corn pollen on a non-target lycaenid butterfly. *Appl. Entomol. Zool.* **2005**, *40*, 151–159.
- (56) Felke, M.; Lorenz, N.; Langenbruch, G. A. Laboratory studies on the effects of pollen from Bt-maize on larvae of some butterfly species. *J. Appl. Entomol.* **2002**, *126*, 320–325.
- (57) Rosi-Marshall, E. J.; Tank, J. L.; Royer, T. V.; Whiles, M. R.; Evans-White, M. A.; Chambers, C. P.; Griffiths, N. A.; Pokelsek, J.; Stephen, M. L. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 16204–16208.
- (58) Chambers, C. P.; Whiles, M. R.; Rosi-Marshall, E. J.; Tank, J. L.; Royer, T. V.; Griffiths, N. A.; Evans-White, M. A.; Stojak, A. R. Responses of stream macroinvertebrates to Bt maize leaf detritus. *Ecol. Appl.* **2010**, *20*, 1949–1960.
- (59) Jensen, P. D.; Dively, G. P.; Swan, C. M.; Lamp, W. O. Exposure and nontarget effects of transgenic Bt corn debris in streams. *Environ. Entomol.* **2010**, *39*, 707–714.
- (60) Swan, C. M.; Jensen, P. D.; Dively, G. P.; Lamp, W. O. Processing of transgenic crop residues in stream ecosystems. *J. Appl. Ecol.* **2009**, *46*, 1304–1313.
- (61) Prihoda, K. R.; Coats, J. R. Aquatic fate and effects of *Bacillus thuringiensis* Cry3Bb1 protein: Toward risk assessment. *Environ. Toxicol. Chem.* **2008**, *27*, 793–798.
- (62) Li, Y.-L.; Du, J.; Fang, Z.-X.; You, J. Dissipation of insecticidal Cry1Ac protein and its toxicity to nontarget aquatic organisms. *J. Agric. Food Chem.* **2013**, *61*, 10864–10871.
- (63) Clark, B. W.; Phillips, T. A.; Coats, J. R. Environmental fate and effects of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *J. Agric. Food Chem.* **2005**, *53*, 4643–4653.
- (64) Lang, A.; Otto, M. A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target Lepidoptera. *Entomol. Exp. Appl.* **2010**, *135*, 121–134.
- (65) Yu, H.-L.; Li, Y.-H.; Wu, K.-M. Risk assessment and ecological effects of transgenic *Bacillus thuringiensis* crops on non-target organisms. *J. Integr. Plant Biol.* **2011**, *53*, 520–538.
- (66) Wie, S. I.; Andrews, R. E.; Hammock, B. D.; Faust, R. M.; Bulla, L. A. Enzyme-linked immunosorbent assays for detection and quantitation of the entomocidal parasporal crystalline protein of *Bacillus thuringiensis* subsp. *kurstaki* and *israelensis*. *Appl. Environ. Microbiol.* **1982**, *43*, 891–894.
- (67) Walschus, U. W. E.; Witt, S.; Wittmann, C. Development of monoclonal antibodies against Cry1Ab protein from *Bacillus thuringiensis* and their application in an ELISA for detection of transgenic Bt-maize. *Food Agric. Immunol.* **2002**, *14*, 231.
- (68) Hori, H.; Takahashi, Y.; Takahashi, M.; Wada, Y. Detection of the *Bacillus thuringiensis* serovar *japonensis* strain *Buibui* protoxin with enzyme-linked immunosorbent assay and its application to detection of the protoxin in soil. *Appl. Entomol. Zool.* **2000**, *35*, 401–411.
- (69) Fejes, Á.; Takács, E.; Fekete, G.; Darvas, B.; Ferguson, B. S.; Saxena, D.; Székács, A. Aquatic effect duration study of Cry4 toxin with immunoassay and *Aedes aegypti* larval biotest. *Aquatic Insects* **2012**, *34*, 207–222.
- (70) Takahashi, Y.; Hori, H.; Furuno, H.; Kawano, T.; Takahashi, M.; Wada, Y. Enzyme-linked immunosorbent assays for rapid and quantitative detection of insecticidal crystal proteins of Bt pesticides. *J. Pestic. Sci.* **1998**, *23*, 386–391.
- (71) Crowther, J. R. *The ELISA Guidebook*, 2nd ed.; Humana Press: New York City, NY, 2009; Vol. 516.
- (72) Lipton, C. R.; Dautlick, J. X.; Grothaus, G. D.; Hunst, P. L.; Magin, K. M.; Mihaliak, C. A.; Rubio, F. M.; Stave, J. W. Guidelines for the validation and use of immunoassays for determination of introduced proteins in biotechnology enhanced crops and derived food ingredients. *Food Agric. Immunol.* **2000**, *12*, 153–164.
- (73) Brown, M. C. Microtiter plate ELISA. In *Immunoassays in Agricultural Biotechnology*; Shan, G., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 59–89.
- (74) Sheedy, C.; Yau, K. Y. Principles of immunoassays. In *Immunoassays in Agricultural Biotechnology*; Shan, G. Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 5–22.
- (75) Stave, J. W. Protein immunoassay methods for detection of biotech crops: applications, limitations, and practical considerations. *J. AOAC Int.* **2002**, *85*, 780–786.
- (76) Packer, B.; Silvanovich, A.; Grothaus, G. D. Immunoassay applications in trait discovery, product development, and registration. In *Immunoassays in Agricultural Biotechnology*; Shan, G., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 219–239.
- (77) Clapper, G. M.; Kurman, L. Immunoassay applications in grain products and food processing. In *Immunoassays in Agricultural Biotechnology*; Shan, G., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 241–256.
- (78) Palm, C. J.; Seidler, R. J.; Schaller, D. L.; Donegan, K. K. Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki*  $\delta$ -endotoxin. *Can. J. Microbiol.* **1996**, *42*, 1258–1262.
- (79) Tapp, H.; Stotzky, G. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biol. Biochem.* **1998**, *30*, 471–476.
- (80) Saxena, D.; Stotzky, G. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. *FEMS Microbiol. Ecol.* **2000**, *33*, 35–39.
- (81) Douville, M.; Gagne, F.; Masson, L.; McKay, J.; Blaise, C. Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. *Biochem. Syst. Ecol.* **2005**, *33*, 219–232.
- (82) Prihoda, K. R.; Coats, J. R. Fate of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein in a soil microcosm. *Chemosphere* **2008**, *73*, 1102–1107.
- (83) Sundaram, K. M. S.; Sundaram, A.; Hammock, B. D. Persistence of *Bacillus thuringiensis* deposits in a hardwood forest, after aerial application of a commercial formulation at two dosage rates. *J. Environ. Sci. Health, Part B* **1994**, *29*, 999–1052.
- (84) Gruber, H.; Paul, V.; Meyer, H. H.; Müller, M. Determination of insecticidal Cry1Ab protein in soil collected in the final growing seasons of a nine-year field trial of Bt-maize MON810. *Transgenic Res.* **2012**, *21*, 77–88.
- (85) Daudu, C. K.; Muchaonyerwa, P.; Mnkeni, P. N. S. Litterbag decomposition of genetically modified maize residues and their constituent *Bacillus thuringiensis* protein (Cry1Ab) under field conditions in the central region of the Eastern Cape, South Africa. *Agric., Ecosyst. Environ.* **2009**, *134*, 153–158.
- (86) Baumgarte, S.; Tebbe, C. C. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Mol. Ecol.* **2005**, *14*, 2539–2551.
- (87) Tank, J. L.; Rosi-Marshall, E. J.; Royer, T. V.; Whiles, M. R.; Griffiths, N. A.; Frauendorf, T. C.; Treering, D. J. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 17645–17650.



- (88) Cheung, P. Y. K.; Gee, S. J.; Hammock, B. D. Pesticide immunoassay as a biotechnology. In *The Impact of Chemistry on Biotechnology*; Phillips, M. P., Shoemaker, S. P., Middlekauff, R. D., Eds.; American Chemical Society: Washington, D.C., 1988; Vol. 362, pp 217–229.
- (89) Agdia Bt-Cry1Ab/1Ac ELISA Kit User Guide. <https://orders.agdia.com/Documents/m172.pdf> (accessed May 4, 2015).
- (90) Agdia Bt-Cry1Ab/1Ac Product Overview. <https://orders.agdia.com/InventoryD.asp?attribute=Size=480&collection=PSP+06200&loc=IN> (accessed May 4, 2015).
- (91) Jung, F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X.; Hammock, B. D. Use of immunochemical techniques for the analysis of pesticides. *Pestic. Sci.* **1989**, *26*, 303–317.
- (92) Shan, G.; Embrey, S. K.; Schafer, B. W. A highly specific enzyme-linked immunosorbent assay for the detection of Cry1Ac insecticidal crystal protein in transgenic WideStrike cotton. *J. Agric. Food Chem.* **2007**, *55*, 5974–5979.
- (93) Schmidt, J.; Alarcon, C. Immunoassay method validation. In *Immunoassays in Agricultural Biotechnology*; Shan, G., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2011; pp 115–138.
- (94) Parker, C. W. *Radioimmunoassay of biologically active compounds*; Prentice-Hall: Englewood Cliffs, NJ, 1976.
- (95) Adrian, J.; Pinacho, D.; Granier, B.; Diserens, J.-M.; Sánchez-Baeza, F.; Marco, M. P. A multianalyte ELISA for immunochemical screening of sulfonamide, fluoroquinolone and  $\beta$ -lactam antibiotics in milk samples using class-selective bioreceptors. *Anal. Bioanal. Chem.* **2008**, *391*, 1703–1712.
- (96) Shan, G.; Embrey, S. K.; Herman, R. A.; McCormick, R. Cry1F protein not detected in soil after three years of transgenic Bt corn (1507 corn) use. *Environ. Entomol.* **2008**, *37*, 255–262.
- (97) Mueting, S. A.; Strain, K. E.; Lydy, M. J. Validation of an extraction method for Cry1Ab protein from soil. *Environ. Toxicol. Chem.* **2014**, *33*, 18–25.
- (98) Strain, K. E.; Whiting, S. A.; Lydy, M. J. Laboratory and field validation of a Cry1Ab protein quantitation method for water. *Talanta* **2014**, *128*, 109–116.
- (99) Li, Y.-L.; Fang, Z.-X.; You, J. Application of Box-Behnken experimental design to optimize the extraction of insecticidal Cry1Ac from soil. *J. Agric. Food Chem.* **2013**, *61*, 1464–1470.
- (100) Schwartz, E.; Mileguir, F.; Grossman, Z.; Mendelson, E. Evaluation of ELISA-based sero-diagnosis of dengue fever in travelers. *J. Clin. Virol.* **2000**, *19*, 169–173.
- (101) Lutz, B.; Wiedemann, S.; Einspanier, R.; Mayer, J.; Albrecht, C. Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. *J. Agric. Food Chem.* **2005**, *53*, 1453–1456.
- (102) Durnez, L.; Van Bortel, W.; Denis, L.; Roelants, P.; Veracx, A.; Trung, H. D.; Sochantha, T.; Coosemans, M. False positive circumsporozoite protein ELISA: a challenge for the estimation of the entomological inoculation rate of malaria and for vector incrimination. *Malar. J.* **2011**, *10*, 195.
- (103) Einspanier, R.; Lutz, B.; Rief, S.; Berezina, O.; Zverlov, V.; Schwarz, W.; Mayer, J. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize. *Eur. Food Res. Technol.* **2004**, *218*, 269–273.
- (104) Emmerling, C.; Strunk, H.; Schöbinger, U.; Schrader, S. Fragmentation of Cry1Ab protein from Bt-maize (MON810) through the gut of the earthworm species *Lumbricus terrestris* L. *Eur. J. Soil Biol.* **2011**, *47*, 160–164.
- (105) Dubelman, S.; Ayden, B. R.; Bader, B. M.; Brown, C. R.; Jiang, C.; Vlachos, D. Cry1Ab protein does not persist in soil after 3 years of sustained Bt corn use. *Environ. Entomol.* **2005**, *34*, 915–921.
- (106) Vettori, C.; Paffetti, D.; Saxena, D.; Stotzky, G.; Giannini, R. Persistence of toxins and cells of *Bacillus thuringiensis* subsp. *kurstaki* introduced in sprays to Sardinia soils. *Soil Biol. Biochem.* **2003**, *35*, 1635–1642.
- (107) Delannay, X.; LaVallee, B. J.; Proksch, R. K.; Fuchs, R. L.; Sims, S. R.; Greenplate, J. T.; Marrone, P. G.; Dodson, R. B.; Augustine, J. J.; Layton, J. G. Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. *Nat. Biotechnol.* **1989**, *7*, 1265–1269.
- (108) Zwahlen, C.; Hilbeck, A.; Gugerli, P.; Nentwig, W. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Mol. Ecol.* **2003**, *12*, 765–775.
- (109) Head, G.; Surber, J. B.; Watson, J. A.; Martin, J. W.; Duan, J. J. No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. *Environ. Entomol.* **2002**, *31*, 30–36.
- (110) Rosenberg, I. M. *Protein Analysis and Purification: Benchtop Techniques*; Birkhäuser: Boston, MA, 1996.
- (111) Gruber, H.; Paul, V.; Guertler, P.; Spiekens, H.; Tichopad, A.; Meyer, H. H.; Müller, M. Fate of Cry1Ab protein in agricultural systems under slurry management of cows fed genetically modified maize (*Zea mays* L.) MON810: a quantitative assessment. *J. Agric. Food Chem.* **2011**, *59*, 7135–7144.
- (112) Paul, V.; Guertler, P.; Wiedemann, S.; Meyer, H. H. Degradation of Cry1Ab protein from genetically modified maize (MON810) in relation to total dietary feed proteins in dairy cow digestion. *Transgenic Res.* **2010**, *19*, 683–689.
- (113) Delahunty, C.; Yates Iii, J. R. Protein identification using 2D-LC-MS/MS. *Methods* **2005**, *35*, 248–255.
- (114) Yang, Q.; Tang, S.; Rang, J.; Zuo, M.; Ding, X.; Sun, Y.; Feng, P.; Xia, L. Detection of toxin proteins from *Bacillus thuringiensis* strain 4.0718 by strategy of 2D-LC–MS/MS. *Curr. Microbiol.* **2015**, *70*, 457–463.
- (115) Bayyareddy, K.; Zhu, X.; Orlando, R.; Adang, M. J. Proteome analysis of Cry4Ba toxin-interacting *Aedes aegypti* lipid rafts using geLC–MS/MS. *J. Proteome Res.* **2012**, *11*, 5843–5855.
- (116) Hu, X. T.; Owens, M. A. Multiplexed protein quantification in maize leaves by liquid chromatography coupled with tandem mass spectrometry: an alternative tool to immunoassays for target protein analysis in genetically engineered crops. *J. Agric. Food Chem.* **2011**, *59*, 3551–3558.
- (117) Gruber, H.; Paul, V.; Meyer, H. H. D.; Müller, M. Validation of an enzyme immunoassay for monitoring Cry1Ab toxin in soils planted with Bt-maize (MON810) in a long-term field trial on four South German sites. *J. Verbraucherschutz Lebensmittelsicherh.* **2009**, *3*, 22–25.
- (118) Ahmad, A.; Wilde, G. E.; Zhu, K. Y. Detectability of coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods. *Environ. Entomol.* **2005**, *34*, 385–394.
- (119) Icoz, I.; Stotzky, G. Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil. *Transgenic Res.* **2008**, *17*, 609–620.
- (120) Badea, E. M.; Chelu, F.; Lacatusu, A. Results regarding the levels of Cry1Ab protein in transgenic corn tissue (MON810) and the fate of Bt protein in three soil types. *Rom. Biotechnol. Lett.* **2010**, *15*, 55–62.
- (121) Miethling-Graff, R.; Dockhorn, S.; Tebbe, C. C. Release of the recombinant Cry3Bb1 protein of Bt maize MON88017 into field soil and detection of effects on the diversity of rhizosphere bacteria. *Eur. J. Soil Biol.* **2010**, *46*, 41–48.
- (122) Helassa, N.; M'Charek, A.; Quiquampoix, H.; Noinville, S.; Déjardin, P.; Frutos, R.; Staunton, S. Effects of physicochemical interactions and microbial activity on the persistence of Cry1Aa Bt (*Bacillus thuringiensis*) toxin in soil. *Soil Biol. Biochem.* **2011**, *43*, 1089–1097.
- (123) Whiting, S. A.; Strain, K. E.; Campbell, L. A.; Young, B. G.; Lydy, M. J. A multi-year field study to evaluate the environmental fate and agronomic effects of insecticide mixtures. *Sci. Total Environ.* **2014**, *497–498*, 534–542.
- (124) Wang, Y.; Hu, H.; Huang, J.; Li, J.; Liu, B.; Zhang, G. Determination of the movement and persistence of Cry1Ab/1Ac protein released from Bt transgenic rice under field and hydroponic conditions. *Soil Biol. Biochem.* **2013**, *58*, 107–114.
- (125) Xue, K.; Diaz, B. R.; Thies, J. E. Stability of Cry3Bb1 protein in soils and its degradation in transgenic corn residues. *Soil Biol. Biochem.* **2014**, *76*, 119–126.

- (126) Wang, H.; Ye, Q.; Wang, W.; Wu, L.; Wu, W. Cry1Ab protein from Bt transgenic rice does not residue in rhizosphere soil. *Environ. Pollut.* **2006**, *143*, 449–455.
- (127) Nguyen, H. T.; Jehle, J. A. Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize Mon810. *J. Plant Dis. Prot.* **2007**, *114*, 82–87.
- (128) Marchetti, E.; Accinelli, C.; Talamè, V.; Epifani, R. Persistence of Cry toxins and cry genes from genetically modified plants in two agricultural soils. *Agron. Sustainable Dev.* **2007**, *27*, 231–236.
- (129) Hopkins, D.; Gregorich, E. Detection and decay of the Bt endotoxin in soil from a field trial with genetically modified maize. *Eur. J. Soil Sci.* **2003**, *54*, 793–800.
- (130) Schrader, S.; Münchenberg, T.; Baumgarte, S.; Tebbe, C. C. Earthworms of different functional groups affect the fate of the Bt-toxin Cry1Ab from transgenic maize in soil. *Eur. J. Soil Biol.* **2008**, *44*, 283–289.
- (131) Nguyen, H. T.; Hunfeld, H.; Meissle, M.; Miethling-Gragg, R.; Pagel-Wieder, S.; Rauschen, S.; Zurbrugg, C.; Eber, S.; Gessler, F.; Romeis, J.; Tebbe, C. C.; Nentwig, W.; Jehle, J. A. Round robin quantitation of Cry3Bb1 using the qualitative PathoScreen ELISA. *IOBC/wprs Bull.* **2008**, *33*, 59–66.
- (132) Shu, Y.; Ma, H.; Du, Y.; Li, Z.; Feng, Y.; Wang, J. The presence of *Bacillus thuringiensis* (Bt) protein in earthworms *Eisenia fetida* has no deleterious effects on their growth and reproduction. *Chemosphere* **2011**, *85*, 1648–1656.